

# **CONTRIBUTION OF ETHNICITY TO SUBGINGIVAL MICROBIAL COLONIZATION**

A Senior Honors Thesis

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By

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## **ABSTRACT**

Introduction: Although it is known that the gingival sulcus contains a complex microbial ecosystem, the role of host-associated colonization factors, especially ethnicity, in determining the composition of this community is not known. Open-ended molecular approaches are comprehensive tools that allow us to compare profiles of microbial communities with several as-yet-uncultivated organisms. Objective: To compare the subgingival microbial profiles of periodontally healthy subjects belonging to four different ethnicities. Methods: 55 periodontally healthy subjects of Caucasian (n=17), African-American (n=14), Hispanic (n=17), and Chinese (n=17) ethnicities were recruited. All subjects were over age 18 without history of systemic disease, pregnancy, and recent or prophylactic antibiotic use. Ethnicity information and subgingival plaque samples were collected. 16S rRNA genes were amplified using polymerase chain reaction with fluorescently labeled broad-range primers and digested using MspI restriction enzyme. Terminal Restriction Fragment Length Polymorphism Analysis (t-RFLP) was used to examine microbial profiles. Non-parametric tests were used for between group comparisons. Results: A statistically significant difference was found in the total peaks measured between African-Americans and Chinese ( $p=0.0165$ ), African-American and Latino ( $p=0.0001$ ), Caucasian and Chinese ( $p=0.0468$ ), and Caucasian and Latino ( $p=0.0005$ , Kruskal-Wallis analysis). Conclusions: There is an association between ethnic preference and the bacterial composition of the health-associated subgingival plaque. However, the effect of shared environment remains to be investigated.

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## **INTRODUCTION**

It is well established that bacteria in dental plaque are the primary etiological agents of chronic periodontitis, a polymicrobial infection that leads to destruction of the structures that anchor the tooth to the jaws. Several factors, notably nutrition and oral hygiene, are known to have an effect on oral bacterial colonization. However, recent studies on other host-associated ecosystems have suggested that several other host-related factors may play a significant role in bacterial colonization. Therefore, it is important to understand the role of host-related bacterial colonization factors in the acquisition of oral bacteria.

It is known that the susceptibility to periodontal disease is different among different ethnicities. For example, the prevalence of Aggressive Periodontitis is significantly higher in African Americans as compared to Caucasians(14). Since bacteria colonize a tooth soon after its eruption to form a stable, health compatible biofilm community(12), it is important to examine if this colonization is influenced by ethnicity.

The microbial constituents of dental plaque have been studied for over 70 years using microscopy, cultivation and molecular methods to characterize bacterial species(5, 12, 17-19, 21). However, these studies relied on phenotypic characteristics for bacterial identification, which provided a limited ability to accurately identify various bacterial species and explore the diversity of this evolving community. More recently, targeted molecular approaches that detect the presence of certain previously identified species have been used to study plaque colonization(19). The limitations of these studies are immediately obvious; since they require prior knowledge of the bacterial species, they are not designed to provide a complete picture of a

complex microbial community with several unknown species and as a result of this, are unable to be quantitative. Most recently, Terminal restriction fragment length polymorphism (T-RFLP) has been used to compare the microbial profiles of several naturally occurring ecosystems.

The aim of the present study is to compare the microbial profiles of the subgingival sulcus among periodontally healthy subjects from four ethnicities- Caucasian, African-American, Latino, and Chinese; using a molecular method for bacterial community profiling.

## **METHODS**

**Subject selection and study design:** Periodontally healthy individuals between 18-40 years of age were recruited from those responding to recruiting campaigns. Subjects were recruited from the population at the Ohio State University using flyers posted throughout the university campus to facilitate recruitment and by word of mouth.

All subjects interested in the study were emailed an exclusion questionnaire. This electronic interview served to exclude subjects who are below 18 years of age and satisfy the exclusion criteria listed below. Subjects who were smokers, undergoing orthodontic therapy, had antibiotic therapy or professional cleaning within the previous 3 months, required antibiotic coverage before dental treatment, pregnant, required the use of immunosuppressant medications, bisphosphonates or steroids, or had diabetes or HIV, or did not meet the ethnicity requirements were excluded from this study.

Qualifying subjects participated in a periodontal examination to ensure that they satisfied the clinical criteria for inclusion into the study. All subjects were examined by calibrated periodontists. Probe depth, attachment levels, gingival and plaque indices were recorded throughout the mouth on 6 sites per tooth using a PCP-UNC 15 probe. Subjects with at least 20 natural non-carious teeth,  $\leq 3$  mm probing pocket depths at all sites (indicative of healthy gums), and a gingival index of  $\leq 1$  (indicative of absence of gingivitis) were selected using this clinical examination.

Each subject who qualified for the study was explained the purpose and procedures of the research. They were given an option to exit the research at this point. If this option was chosen, all data collected during initial screening was destroyed. Informed consent and HIPPA regulations were also explained. Once the informed consent was obtained, a detailed history including information about ethnicity, education, income, age, sex and medical status including pregnancy and oral contraceptive use in the case of females was obtained from each patient.

**Sample collection and DNA isolation:** Subgingival plaque was collected using Endodontic paperpoints (Caulk Dentsply) inserted into the mesial sulcus of every sampled tooth in the mouth. All the paper point and scaler samples were pooled. Gingival crevicular fluid (GCF) was collected using Periopaper™ strips (Oraflow, Plainview, New York, USA) inserted into the sulcus for 30s, and stored in liquid nitrogen. 8 strips were collected from each subject. Samples were placed in 1.5-ml microcentrifuge tubes and frozen until further analysis. Bacteria were removed from the subgingival sampling devices by adding 200 $\mu$ l of phosphate buffered saline to the tubes, and vortexing. The sampling devices were then removed, and DNA isolated with a

Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA) using the tissue protocol according to the manufacturer's instructions.

**t-RFLP analysis:** Bacterial 16S rRNA genes were amplified using 22 cycles of PCR with fluorescent- labeled broad range bacterial primers A18-FAM (5'- TT TGA TCC TGG CTC AG- FAM-3') and 317-HEX (5'- FAM-AAG GAG GTG ATC CAG GC -3') (Applied Biosystems, Foster City, CA). The cycling conditions have previously been described(10). The amplicons were purified using a Qiaquick kit (Qiagen, Valencia, CA). Restriction digestion was carried out with 5µl of purified PCR product and 10 U of *Msp I* in a total volume of 10µl at 37°C for three hours. 10ul of the restriction digestion product was purified by AMPure beads (Agencourt Bioscience Corporation, Beverly, MA) according to the manufacturer's protocol and eluted with 50µl water. 5µl of the purified product was denatured with 10µl of deionized formamide and mixed with 0.2µl GeneScan 1200 LIZ size standard (Applied Biosystems, Foster City, CA). Fragment lengths were determined on an AB 3730 DNA Analyzer in GeneScan mode. The number of peaks as well as the height and area of each peak; reflecting the sizes and intensities of the terminal fragments were determined using the GeneMapper 4.0 Software.

**Data analysis:** Fragments with a peak height of less than 25 fluorescence units were excluded from analysis. Peak areas were standardized by converting the raw values to a proportion of the total area as previously described(20). Peaks representing less than 1% of the total area were assigned a value of zero and the percentages of the remaining peaks recalculated.

The total numbers of peaks as well as the number of shared peaks were compared between each subject. The peak area was used to compute the Shannon-Weiner diversity index.

Statistical analysis was carried out with JMP (SAS Institute Inc., Cary, NC). The clinical features were compared using ANOVA and non-parametric tests were used to compare bacterial parameters among the different ethnicities.

## **RESULTS**

Clinical and laboratory data was collected from 55 total samples. The ages of the participants is shown in Figure 1. 14 African-American subjects had an age range of 18-38 with a mean of  $27.7 \pm 6.3$  years, 17 Caucasian subjects had an age range 19-22 with a mean of  $20.5 \pm 0.9$  years, 17 Chinese subjects had an age range 20-42 with a mean of  $26.3 \pm 4.2$  years, and 17 Latino subjects had an age range 21-32 with a mean of  $26.2 \pm 2.2$  years. Caucasians were significantly younger than the other three ethnicities ( $p=0.01$ , ANOVA).

Figure 2 shows the amount of supragingival plaque in each individual's mouth, as measured by the Loe and Silness plaque index. Plaque index was  $0.3 \pm 0.4$  for African-Americans,  $1.4 \pm 0.7$  for Caucasians,  $0.8 \pm 0.8$  for Chinese, and  $0.9 \pm 0.8$  for Latino subjects. There was no statistically significant difference found in plaque index between the four ethnicities ( $p>0.05$ , ANOVA).

The total number of peaks in each of the four ethnicities is shown in Figure 3. The mean number of peaks denoting the number of species in each subject was  $42.5 \pm 19.9$  for African-Americans,  $56.9 \pm 18.0$  for Caucasians,  $108.6 \pm 18.0$  for Chinese, and  $151.4 \pm 18.0$  for Latinos. A statistically

significant difference was found in the total peaks measured between African-Americans and Chinese ( $p=0.0165$ ), African-American and Latino ( $p=0.0001$ ), Caucasian and Chinese ( $p=0.0468$ ), and Caucasian and Latino ( $p=0.0005$ , Kruskal-Wallis analysis).

The number of individuals within an ethnic group who shared one or more species is shown in Figure 4. Values above the blue line indicate the number of species shared by 50% or more individuals within an ethnic group. Latinos shared 117 species, while Caucasians shared 15 species, African Americans shared 23 species, and Chinese shared 59 species. These differences were statistically significant ( $p<0.05$ , Kruskal Wallis Analysis).

A Shannon Weiner diversity index, denoting the total number of species and the abundance of each species within the community, is shown in Figure 5. The mean diversity index was  $3.9\pm0.8$  for African-Americans,  $3.3\pm1.1$  for Caucasians,  $4.6\pm1.5$  for Chinese, and  $3.7\pm1.3$  for Latinos. The subgingival microbial profile of Chinese is significantly higher than Caucasian ( $p=0.0032$ ) and Latino ( $p=0.0454$ ).

The gingival crevicular fluid (GCF) levels are shown in Figure 6. The mean gingival crevicular fluid levels were  $58.2\pm29.7\mu\text{l}$  for African-Americans,  $80.7\pm37.3$  for Caucasians,  $79.8\pm29.4$  for Chinese, and  $69.1\pm31.0$  for Latino subjects. There was no statistically significant difference found in the amount of gingival crevicular fluid collected between the four ethnicities ( $p>0.05$ , ANOVA).



## **DISCUSSION**

This study was designed to examine the role played by ethnicity in determining subgingival bacterial colonization. African-American, Caucasian, Chinese, and Latino subjects were chosen to participate after meeting the inclusion criteria. The four ethnicities chosen were selected because they represent the four major ethnicities in the United States. These four ethnic groups are all characterized by a distinct diet and environment. The age range of the subjects selected for the study was 18-40, because it has been noted that after 40 an increase in the rate of periodontal disease is observed(2). Limiting the age to 18-40 years of age ensured that there was still some diversity among the population, but ruled out any differences in health due to age, as individuals in this age range are generally regarded to be at the same developmental stage in oral health.

Terminal restriction fragment length polymorphism analysis (t-RFLP) of the 16S rRNA gene is an open-ended molecular approach that has been used as a high-throughput tool to compare several naturally occurring microbial communities(7, 16, 20, 23, 24). t-RFLP generates a unique ‘fingerprint’ of each microbial community based on 16S sequence variations among different bacterial species, providing quantitative information on the compositional differences between communities. Terminal restriction fragment length polymorphism (T-RFLP) analysis measures the size polymorphism of terminal restriction fragments from a PCR amplified population(15). It provides a rapid and sensitive technique for assessing diversity within a bacterial community as well as comparative distribution across communities. (15). Numerous studies have used T-RFLP for comparative community analysis in many different fields such as comparison of oral bacterial

microflora in saliva of healthy and patients of periodontitis, comparison between human from cow fecal contamination and between bacterial communities of plant species(1, 16, 23, 24). T-RFLP has been shown to be useful for the assessment of the diversity of human microflora and other bacterial communities (3, 13, 22). Bacterial dynamics and evolutionary diverge between species have also been successfully assessed with the use of T-RFLP(6, 8, 9).

Detection sensitivity of this method is high and comparable to other DNA-based community analysis techniques but potential errors in the process might be attributed to pipetting, sampling method(4). Furthermore, low DNA purity results towards lower diversity and consequently in low reproducibility of T-RFLP profiles and lower DNA yield does not lead to a bias towards lower diversity(11).

Digestion of the 16S rRNA gene using restriction enzymes is sequence-specific, generating terminal fragments of varying lengths due to sequence variations among different bacterial species. Thus, the total number of peaks represents the number of unique species present in the community. The present study detected fewer species in African Americans and Caucasians (Figure 3), suggesting that ethnicity does affect the number of species that colonize the biofilm. This was evident despite the fact that Caucasians were significantly younger than the African-Americans. However, it has been suggested that t-RFLP may underestimate the number of species in a sample, since closely related species may share common restriction sites(13). Further, species of low abundance may not be consistently represented in the t-RFLP profile(13). Hence, if ethnicity is attributed to colonization by several closely related species or by species that are less abundant in the community, these results may not be apparent using t-RFLP. An

investigation into the contribution of ethnicity on the prevalence and levels of specific bacterial species using sensitive, targeted molecular approaches is warranted.

Peak area is an estimate of species abundance, since it is a measure of the number of fragments of a particular size. A comparison of the peak areas of the different ethnicities using the Shannon Weiner Diversity index reveals that the greatest diversity was found among the Chinese group, followed by the African Americans, Latinos and Caucasians (Figure 5). This is evident when the amount of plaque and the gingival health in each subject was not significantly different. Taken together, these findings suggest that qualitative differences exist in a health compatible biofilm. This has important implications for the science of probiotics, since microbial replacement therapy for the treatment of periodontal disease must take into consideration the ethnicity of the subject.

In summary, there are significant differences in the subgingival bacterial profiles of individuals belonging to different ethnicities. It is not within the scope of this study to examine what species are predominant in each ethnic group, however, such information would provide valuable insight into the disease susceptibility of each ethnic group. It is also not known from this study, how ethnicity contributes to this difference in bacterial colonization. Food and nutritional habits as well as genetic differences may play a role and deserve further investigation.

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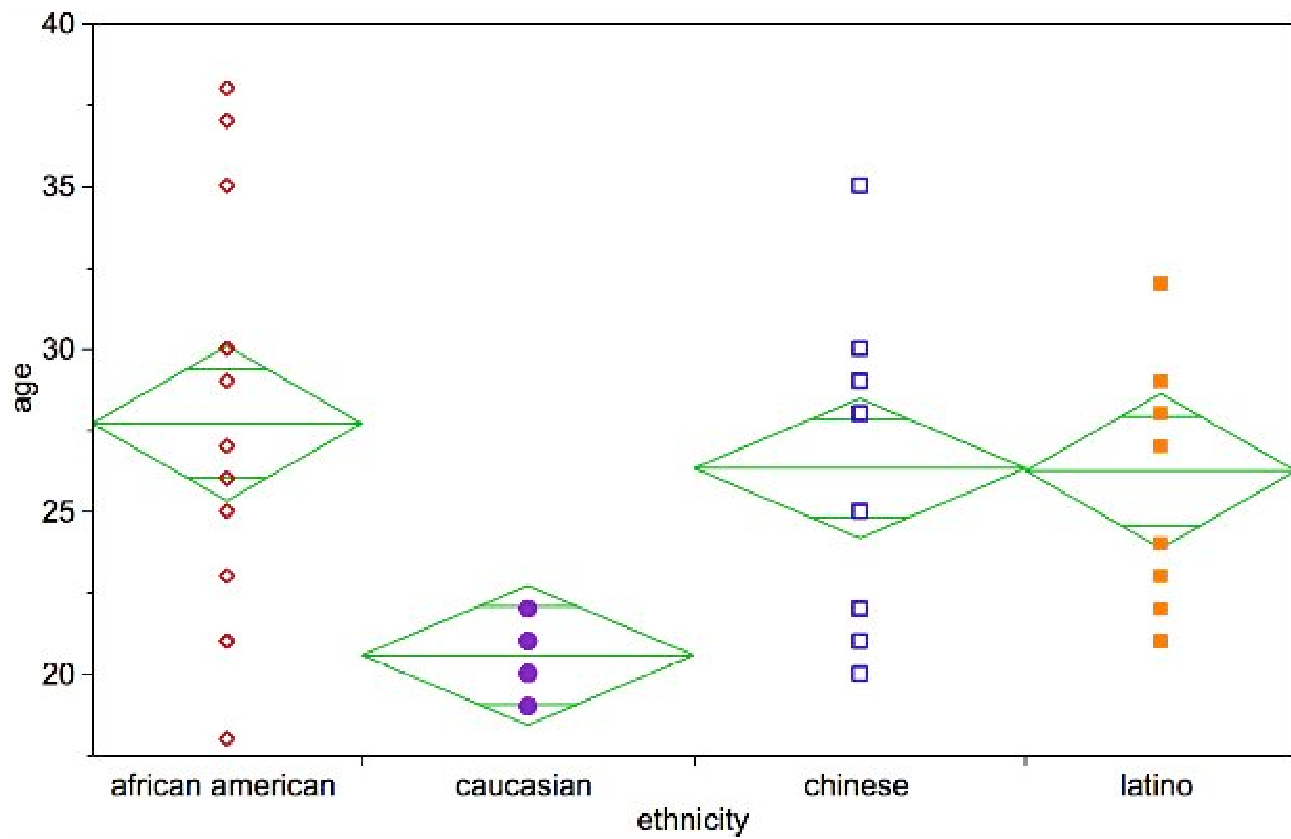


Figure 1. Age of all participating subjects by ethnicity. Caucasians mean age was significantly lower than the other three ethnic groups ( $p > 0.01$ , ANOVA).

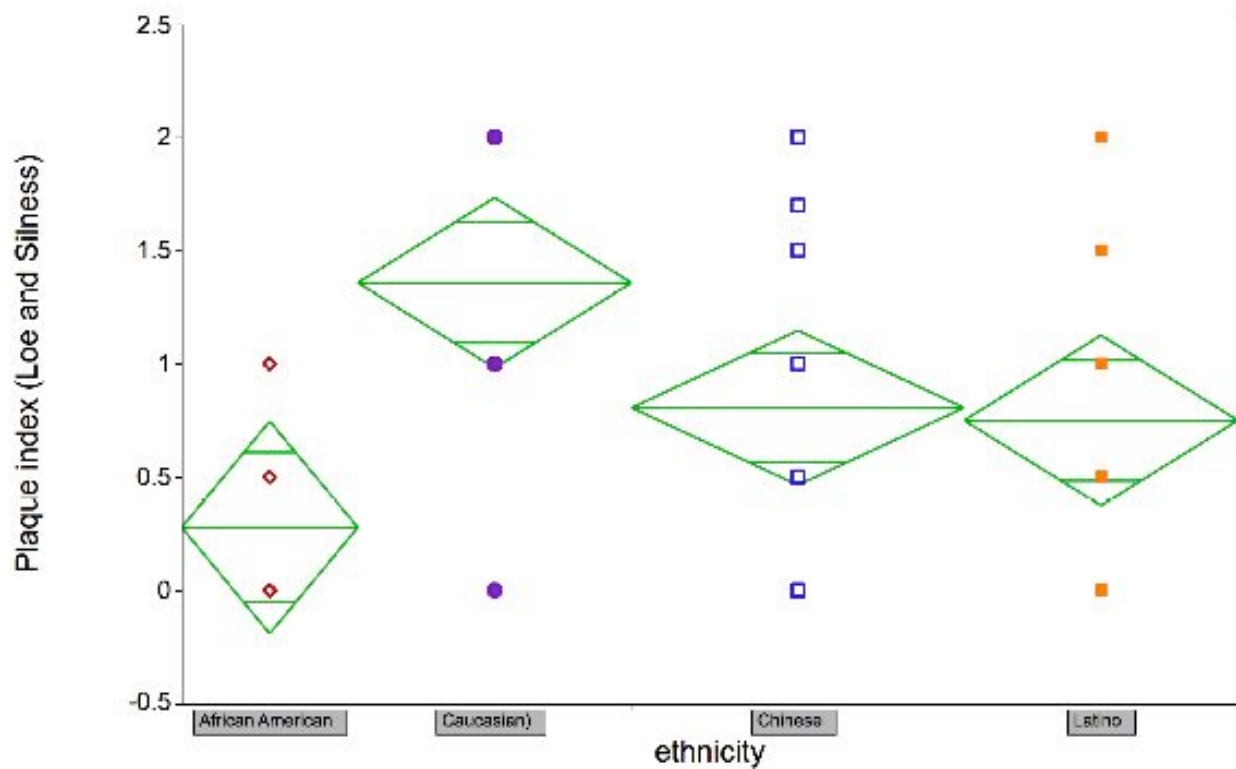


Figure 2. Plaque index of all participating subjects by ethnicity. There were no significant differences in the mean plaque indexes among the four ethnicities by Kruskal-Wallis analysis.

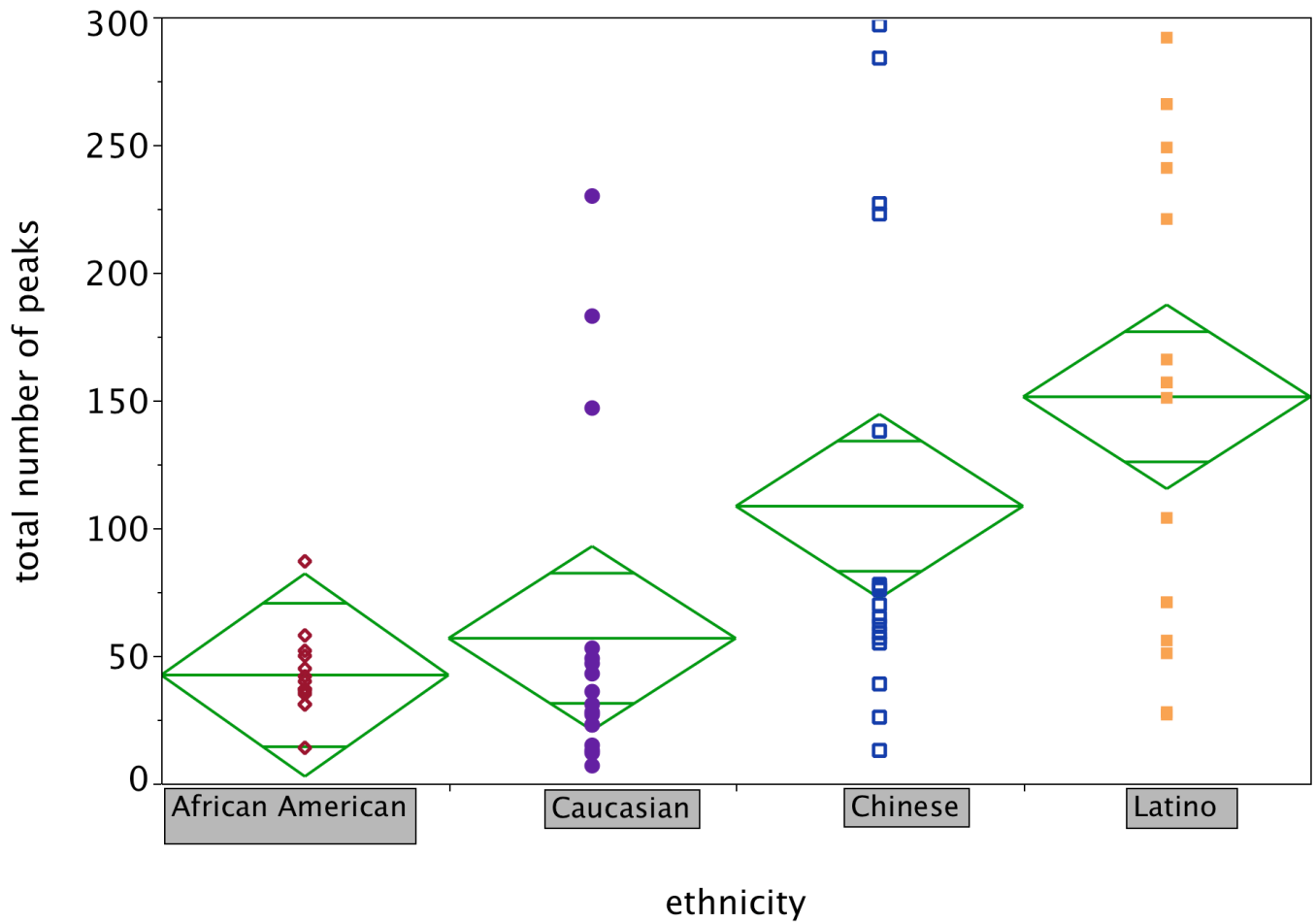


Figure 3. Total number of peaks in subjects by ethnicity. The total number of peaks with a fluorescence greater than 25 units was computed in each subject. There was a statistically significant difference found in the total peaks measured between African-Americans and Chinese ( $p=0.0165$ ), African-American and Latino ( $p=0.0001$ ), Caucasian and Chinese ( $p=0.0468$ ), and Caucasian and Latino ( $p=0.0005$ ) by Kruskal-Wallis analysis.



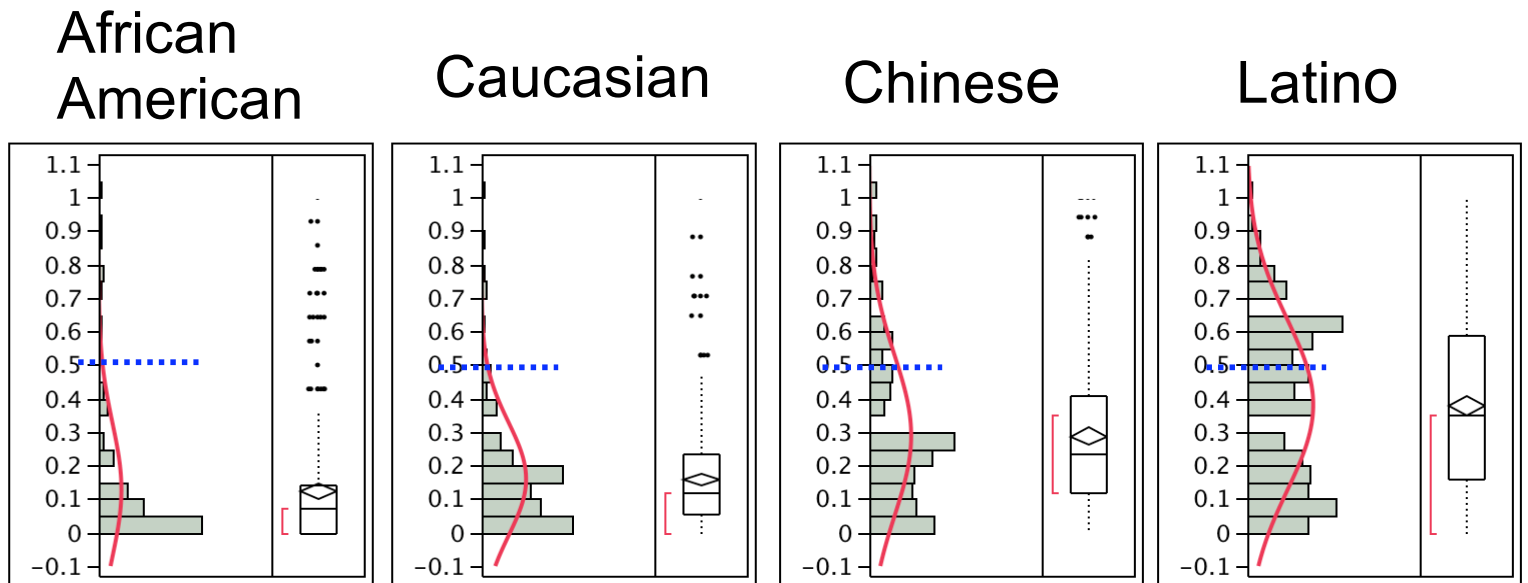


Figure 4. Distribution of species common to all individuals within an ethnic group. Values above the blue line indicate the number of species shared by 50% or more individuals within an ethnic group. These differences were statistically significant ( $p < 0.05$ , Kruskal Wallis Analysis).

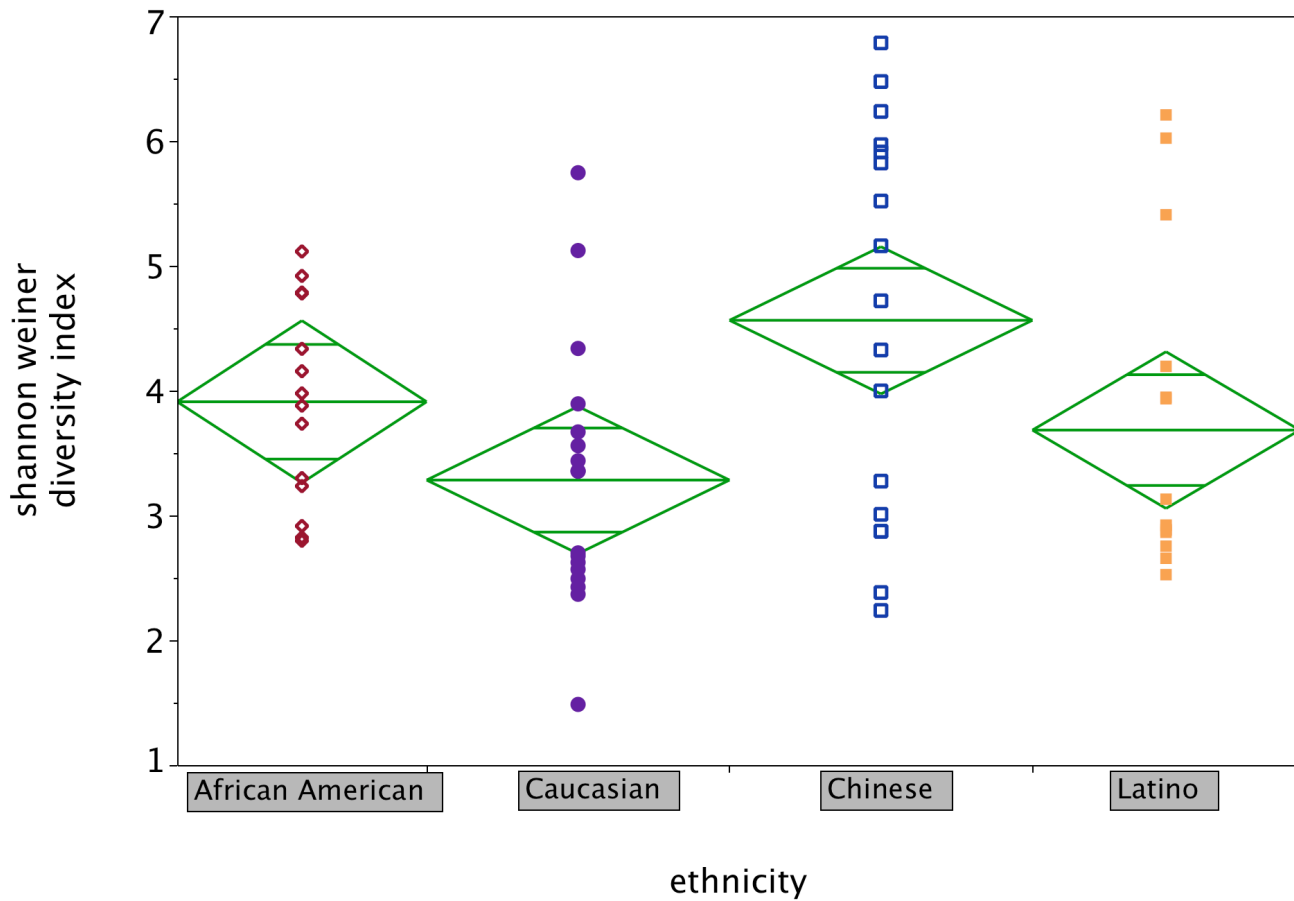


Figure 5. Shannon Weiner diversity index by ethnicity. The index is indicative of the total number of species present and the proportion of each species present compared among subjects. Chinese had a significantly greater bacterial diversity than Caucasian ( $p=0.0032$ ) and Latino ethnicities ( $p=0.0454$ ).

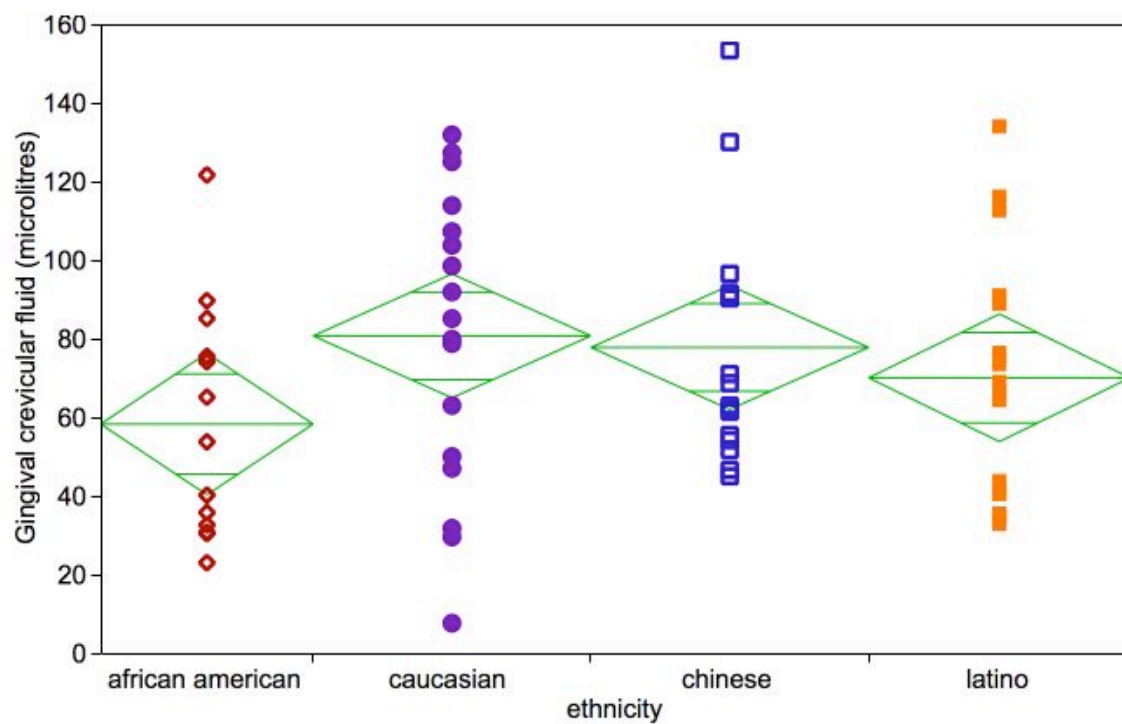


Figure 6. Relationship between GCF flow readings in micro liters and ethnicity. There was no statistically significant difference found in the amount of gingival crevicular fluid collected between the four ethnicities